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<b>(21) International Application Number:</b> PCT/GB95/00730 <b>(22) International Filing Date:</b> 30 March 1995 (30.03.95) <b>(30) Priority Data:</b> 9406371.6 30 March 1994 (30.03.94) GB <b>(71) Applicant (for all designated States except US):</b> AXIS GENETICS LIMITED [GB/GB]; Babraham, Cambridge CB2 4AZ (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> GEOGHEGAN, Irene [GB/GB]; Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA (GB). ROBERTSON, Walter [GB/GB]; Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA (GB). BIRCH, Nicholas [GB/GB]; Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA (GB). GATEHOUSE, Angharad, Margaret, Roscoe [GB/GB]; Department of Biological Sciences, University of Durham, South Road, Durham DH1 3LE (GB). <b>(74) Agent:</b> ELKINGTON AND FIFE; Prospect House, 8 Pembroke Road, Sevenoaks, Kent TN13 1XR (GB).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> NEMATICIDAL PROTEINS  <b>(57) Abstract</b>  The use of lectins derived from Amaryllidaceae, Alliaceae, or Viciaeae for the control of nematodes, in which said use may be either direct or via transgenic plant expression, and a method therefor.		

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## Nematicidal Proteins

### Background of the Invention

The present invention relates to the control of nematode pests.

There are nematode parasites of plants and animals, including humans. The plant parasites can cause significant economic losses in sub-tropical, tropical and temperate agriculture. Plant-parasitic nematodes are small (generally 100-300  $\mu\text{m}$  long but up to 4mm long, and 15-35  $\mu\text{m}$  wide) worm-like animals which feed on root, stem or leaf tissues of living plants. Nematodes are present wherever plants are cultivated. Ectoparasitic nematodes, such as the dagger (*Xiphinema* and *Longidorus* spp.), stubby-root (*Trichodorus* and *Paratrichodorus* spp.) and spiral (*Scutellonema* and *Helicotylenchus* spp.) nematodes, live outside the plant and pierce the plant cells with their stylet in order to feed. Migratory endoparasitic nematodes, such as the lesion (*Pratylenchus* spp.), stem and bulb (*Ditylenchus* spp.) and burrowing (*Radopholus* spp.) nematodes, live and feed inside the plant, migrating through the plant tissues. Sedentary endoparasitic nematodes, such as the root-knot (*Meloidogyne* spp.), cyst (*Globodera* and *Heterodera* spp.), citrus (*Tylenchulus* spp.) and reniform (*Rotylenchulus* spp.) nematodes, live and feed inside the plant, inducing specialised fixed feeding sites called giant cells, syncytia or nurse cells in susceptible plants. Such fixed feeding sites serve as food transfer cells for the various developmental stages of the nematodes. Syncytia originate in the pericycle, endodermis or adjacent cortex.

Various methods have been used to control plant parasitic nematodes. They include quarantine measures, manipulation of planting and harvesting dates, improved fertilization and irrigation

programmes that lessen plant stresses, crop rotation and fallowing, use of resistant and tolerant cultivars and rootstocks, organic soil amendments, and physical (eg solarization), biological and chemical control. Although quarantines are useful, especially when an infestation is first discovered, they are very expensive measures and usually cannot prevent the spread of nematodes. Furthermore, biological control is difficult to manage, and high quantities and repeated additions of agents are required.

Today, control of plant-parasitic nematodes relies mainly on chemical control. Nematicides used commercially are generally either fumigants (eg halogenated aliphatic hydrocarbons and methyl isothiocyanate precursor compounds) or non-fumigants (eg organophosphates and oxime-carbamates). However, the use of chemical nematicides is undesirable because these chemicals are highly toxic and therefore present a hazard to the user and to the environment.

Thus, there is today a real need to have new, more effective, and safe means to control plant-parasitic nematodes.

Using the modern techniques of recombinant DNA and plant genetic engineering, genes encoding nematode control proteins may be cloned and introduced into cells of the appropriate crop plant, where their expression renders that plant inherently resistant to nematode attack. Genetic engineering overcomes the problem of reproductive barriers to genetic recombination.

WO 93/06710 (North Carolina State University) discloses an approach to imparting nematode resistance to plants which comprises transforming plants with a heterologous DNA construct consisting of a plant promoter, which is activated by a nematode attacking the plant, and a structural gene, which encodes a product which is toxic to the plant cells which form the feeding site of the nematode. Examples of products toxic to plant cells which are disclosed are

nucleases, proteinases, toxins from plant pathogenic bacteria, lipases, membrane channel proteins and antibodies which bind to plant cell components. The disadvantage of this approach is that expression of the toxin gene must be restricted to the nematode feeding site in order to prevent death of plant cells in adjacent tissues. In practice this is difficult to achieve.

WO 92/04453 (The University of Leeds) discloses a method for conferring nematode resistance on plants by transforming plants with a heterologous DNA construct comprising a plant promoter, which is induced by nematode infection, and a structural gene encoding a product which is toxic to the plant cells forming the feeding site of the nematode or to the nematode itself. Examples of toxic products which are disclosed are enzymes such as DNase, RNase or a proteinase, antisense RNA, *Bacillus thuringiensis* proteins having anti-nematode activity, or an antibody which disrupts ingestion or digestion of food by the nematode. Such an approach has the disadvantage that it is ineffective against plant parasitic nematodes which do not induce the formation of specialised feeding sites.

WO 92/21757 (Plant Genetic Systems N.V.) discloses a method for conferring nematode resistance on plants which comprises transforming plants with two chimaeric genes. The first chimaeric gene comprises a nematode-induced promoter and a structural gene encoding a toxic product which kills the plant cells of the nematode feeding site or the nematode itself. The second chimaeric gene comprises a nematode-repressed promoter and a structural gene encoding a product which, when expressed in cells of the plant inhibits or inactivates the toxic product of the first chimaeric gene. Examples of types of gene products which kill plant cells or nematodes include nucleases, proteases, antisense DNA, *B. thuringiensis* toxins, collagenases, chitinases, glucanases, peroxidases, superoxide dismutases, lectins, glycosidases, antibacterial peptides, gelatinases, enzyme inhibitors or neurotoxins. Specific examples of gene products which can kill or disable nematodes are not disclosed. A disadvantage of this method is that it

requires transformation of plants with two chimaeric genes, each of which must be expressed only in specific tissues. In practice this is difficult to achieve.

WO 92/15690 (Nickerson Biocem Ltd) discloses proteinase inhibitors that have anti-nematode activity and therefore can be used to protect plants against nematodes, either by delivery of the proteinase inhibitor to nematodes or by transformation of plants with a gene coding for a proteinase inhibitor. Tests on potato plants transformed with a cowpea trypsin inhibitor (CpTI) gene, and in which detectable quantities of CpTI could be measured, were found to have quantifiable effects on the rate of growth and sex ratio of cyst nematodes, and on egg numbers of root-knot nematodes but it was not demonstrated whether these effects were sufficient to reduce crop yield losses due to nematodes.

WO 92/15690 also describes tests on potato plants transformed with a pea lectin gene. Such transformed plants had little or no significant effect on cyst nematode establishment and maturation when compared to non-transformed plants.

Thus the known nematode control genes code for products which are either only partially effective or are non-selective and therefore their utilisation requires the use of additional genes to protect non-target plant cells.

Lectins are a heterogeneous class of (glyco) proteins grouped together based upon their ability to recognize and bind carbohydrate moieties of glycoconjugates. Chitin, the principal structural carbohydrate of insects, is a polymer of N-acetyl glucosamine (GluNAc) and various lectins with sugar binding specificities for GluNAc have been disclosed with insecticidal activity against certain agricultural pests.

EP-A-0351924 (Shell Internationale Research Maatschappij B.V.) relates to a transgenic plant comprising a lectin gene expressing a lectin within the plant foreign to the plant as found in nature. In particular, it discloses that pea lectin has been inserted into tobacco, and the transgenic plant has some degree of insect resistance. EP-A-0427529 (Pioneer Hi-Bred International, Inc) discloses that selected plant lectins have been found to be larvicidal against a number of common insect pests or agricultural crops.

Many lectins are known to be toxic to mammals and birds. For example, the lectins of *Phaseolus vulgaris* are poorly digested by rats and thus are able to react with intestinal cells causing disruption of the brush borders of duodenal and jejunal enterocytes. As a result, abnormal absorption of potentially harmful substances occurs, leading to severe toxic effects. There is a need, therefore, to identify lectins which are toxic to nematodes but at the same time do not exhibit toxicity to mammals or birds. These would be useful in crop protection applications without restriction on the food use of the material in which the foreign lectin is to be presented. WO 92/02139 (Agricultural Genetics Company Ltd) discloses that a group of lectins, characterised by specific mannose-binding ability, in particular derived from Amaryllidaceae and Alliaceae, are effective for the control of insect pests, but are non-toxic to mammals and birds. WO 91/06311 (Scottish Crop Research Institute) discloses that mannose-specific lectins obtained from Amaryllidaceae have anti-viral activity against RNA viruses such as Human Immunodeficiency Virus.

### Summary of the Invention

We have surprisingly found that two groups of lectins, one group characterised by specific mannose-binding ability and in particular derived from Amaryllidaceae or Alliaceae, and the second group characterised by ability to bind mannose, as well as some other sugars, and in particular derived from Viciaeae, are effective for the control of nematode pests, but are non-toxic to mammals and birds.

In its broadest aspect this invention comprises the use of lectins having specific mannose-binding ability and/or derived from Amaryllidaceae or Alliaceae, or having mannose-binding ability and derived from Viciaeae for the control of nematode pests. Specifically, such lectins are presented to nematodes in amounts likely to cause mortality, reduced larval weight and/or delayed development. As a result of the presentation of such lectins to nematode pests of plants, plants may be protected from damage to roots, stems, tubers, and other useful parts. Such lectins are, on the other hand, non-toxic to mammals and constitute a safer alternative to the use of chemical nematicides.

The lectins used according to this invention exhibit mannose-binding properties. Lectins from Alliaceae strongly resemble those of Amaryllidaceae with respect to their molecular structure, carbohydrate binding specificity, amino acid composition and serological properties. All bind D-mannose exclusively. All contain high amounts of acidic and hydroxylic amino acids, glycine and leucine. All contain subunits of Mr 11,500-14,000, not linked by disulphide bonds and may occur as dimers (eg garlic) or tetramers (eg snowdrop). Generally, lectin concentration is higher in bulbs of Amaryllidaceae than it is in bulbs of Alliaceae.



A preferred use of Amaryllidaceae, Alliaceae, and Viciae lectins according to the invention is to insert the genes encoding these proteins into plants.

Various methods are available to those skilled in the art for the introduction and expression of foreign genes in transgenic plants. These include *Agrobacterium*-mediated gene transfer, microinjection of DNA into cells or protoplasts, DNA transfer via growing pollen tubes, DNA uptake by imbibing zygotic embryos, silicon carbide fibre-mediated delivery, microprojectile bombardment (biolistic transfer) and direct DNA uptake employing polyethylene glycol, liposomes or electroporation. Once a line of transgenic plants is established the character may be transferred to other cultivars by conventional plant breeding.

Plants which can be protected, preferably by transformation, according to the methods of this invention include, but are not limited to: rice, wheat, maize, cotton, potato, sugarcane, grapevines, cassava, sweet potato, tobacco, soyabean, sugar beet, beans, banana, tomato, lettuce, oilseed rape and sunflower.

Lectins useful in nematode control and the corresponding genes can be obtained from, but are not necessarily limited to, *Allium sativum* (garlic), *Allium vineale*, *Allium ursinum*, *Allium moly*, *Allium cepa*, *Allium porrum*, *Narcissus pseudonarcissus*, *Clivia miniata*, *Galanthus nivalis* (snowdrop), *Hippeastrum hybr*, *Cicer spp.*, *Lens culinaris*, *Lathyrus odoratus* and *Pisum sativum* (pea).

Alternatively, these proteins may be administered or co-administered directly to plants using an agrochemical formulation or as part of a pesticidal formulation which may also include *Bacillus thuringiensis* (Bt), Bt toxin, or other nematicidal substances.

Nematodes to be controlled include plant parasites belonging to the Orders Dorylaimida and Tylenchida. Nematodes of the Order Dorylaimida which may be controlled by this invention include, but are not limited to, nematodes which vector plant viruses and belong to the Family Longidoridae, for example *Xiphinema* spp. and *Longidorus* spp., or the Family Trichodoridae, for example *Trichodorus* spp. and *Paratrichodorus* spp. Nematodes of the Order Tylenchida which may be controlled by this invention include, but are not limited to: migratory ectoparasites belonging to the Families Anguinidae, for example *Ditylenchus* spp., Dolichodoridae, for example *Dolichodorus* spp., and Belenolaimidae, for example *Belenolaimus* spp. and *Trophanus* spp.; obligate parasites belonging to the Families Pratylenchidae, for example *Pratylenchus* spp., *Radopholus* spp. and *Nacobbus* spp., Hoplolaimidae, for example *Helicotylenchus* spp., *Scutellonema* spp. and *Rotylenchulus* spp., Heteroderidae, for example *Heterodera* spp., *Globodera* spp., *Meloidogyne* spp. and *Meloinema* spp., Criconematidae, for example *Croconema* spp. and *Hemicycliophora* spp., and Tylenchulidae, for example *Tylenchulus* spp., *Paratylenchulus* spp. and *Tylenchocriconema* spp.; and parasites belonging to the Families Aphelenchoididae, for example *Aphelenchoides* spp., *Bursaphelenchus* spp. and *Rhadinaphelenchus* spp., and Fergusobiidae, for example *Fergusobia* spp.

#### **Extraction of Lectins from Plant Material**

For the purpose of extracting lectins from Amaryllidaceae and Alliaceae species, such as *Narcissus pseudonarcissus* and *Galanthus nivalis*, the following procedure may be followed.

The bulbs or leaves are homogenized with a blender using 50 ml of 1M ammonium sulphate per gram of fresh tissue. Afterwards the extract is filtered through cheese cloth and centrifuged (4,000 g for 10 minutes). The resulting supernatant is frozen overnight at -20°C. After thawing, the

precipitate is removed by a second centrifugation. The clarified supernatant is applied to a column of mannose-Sepharose (50 ml bed volume) equilibrated with 1M ammonium sulphate. Unbound proteins are washed off and lectin is desorbed using unbuffered 20mM 1,3-diaminopropane.

To remove all phenolic compounds, the affinity-purified lectin is brought up to 1M ammonium sulphate by adding the solid salt, and applied to a column of phenyl Sepharose (Trade-mark) (15 x 3 cm) equilibrated with 1M ammonium sulphate. After washing the column, lectins are eluted using distilled water or 1,3-diaminopropane (20 mM, unbuffered solution).

The lectin from pea may be prepared by a saccharide affinity method based on that of Blobbel and Dobberstein [J. Cell Biology (1975) 67, 835-851] for the preparation of concanavalin A. Mature cotyledons of *Pisum sativum* cv 'Feltham First' were blended in a food processor in 20 mM N-tris[hydroxymethyl]methyl-2-amino-ethanesulphonic acid, 0.5M NaCl, 0.1mM phenylmethylsulphonyl fluoride, pH7.5. Insoluble material was removed by centrifugation at 10,000 x g for 30 minutes. The supernatant was made 30% w/v with respect to ammonium sulphate by addition of the solid salt and clarified by centrifugation as above. The supernatant was made 80% w/v with respect to ammonium sulphate by addition of the solid salt and the precipitated material collected by centrifugation, dissolved in distilled water and dialysed first against water and then against 1M NaCl. The dialysate was mixed with Sephadex™ G-50 which had previously been equilibrated with 1 M NaCl. The slurry was formed into a column and washed through with 1 M NaCl for 48 hours. The lectin was displaced from the column with 0.2 M D-glucose in 1 M NaCl.

### Cloning of Lectin Genes for Insertion into Plants

The cloning of genes for Amaryllidaceae and Alliaceae lectins poses special problems. Extraction of RNA from bulb tissues is particularly difficult. It has been found that ovary tissue, where lectins have been found to be abundant, is suitable for the extraction of mRNA.

The following describes a method for obtaining lectin genes from snowdrop (*Galanthus nivalis*). Those skilled in the art would know that this protocol could be adapted easily for other members of Amaryllidaceae or Alliaceae.

Flowering plants of snowdrop are collected and the ovaries excised from the flowers, frozen in liquid nitrogen and stored at -80°C. Total cellular RNA is prepared from ovary tissue essentially as described by Finkelstein and Crouch [Plant Physiology (1986) 81, 907-912]. Poly A rich RNA is purified by chromatography on oligo-deoxythymidine cellulose as described by Siflow et al [Biochemistry (1979) 18, 2725-2731] except that poly A rich RNA is eluted at room temperature.

A cDNA library can be made using the poly A-enriched RNA isolated using a cDNA synthesis kit and inserted into the *EcoRI* site of a multifunctional phagemid pT7T3 18U. The library is propagated in *E. coli* XL1 Blue.

In order to select clones recombinant for the lectin gene, the colonies are screened using a <sup>32</sup>P-end-labelled partially degenerate oligonucleotide probe derived from the amino acid sequence of the lectin for residues 41-45 ie:-

```
5'   TGT   GTT   TGT   TGC   CCA   3'
5'   TGT   GTT   TGT   AGC   CCA   3'
5'   TGT   GTT   TGT   GGC   CCA   3'
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Hybridisation is carried out for 12 hours at 38°C in 0.9M sodium chloride containing 90 mM Tris-HCl pH 7.5, 6 mM EDTA, 10 x Denhardts, 0.1% SDS, 180 mg/ml hydrolyzed yeast RNA and  $2 \times 10^6$  cpm/ml  $^{32}\text{P}$ -labelled probe. After hybridisation, filters are washed four times in 6 x SSC (1 x SSC = 0.9 M sodium chloride and 0.09 M sodium citrate, pH 7.0) at room temperature for 15 minutes followed by a 5 minute wash at hybridisation temperature in 6 x SSC. Filters are blotted dry, wrapped in Saran Wrap and exposed to Kodak-X-Omat film at -80°C. Colonies producing positive signals are rescreened using the same probe under the same conditions. Plasmids are isolated from purified colonies using the alkaline lysis method as described by Birnboim and Doly [Nucleic Acids Research (1979) 7, 1513-1522] and sequenced to identify the lectin gene using the dideoxy method as described by Sanger et al [Proc. Natl. Acad. Sci. (1977) 74, 5463-5467].

Complete nucleotide sequences for cDNA's corresponding to several isoforms of snowdrop lectin are shown in the accompanying sequence listings. The lectin cDNA LECGNA2 contains an open reading frame of 570 nucleotides with a probable initiation codon at position 18. Translation starting with this codon generates a 157 amino acid polypeptide with a calculated molecular weight of 16,917 daltons that corresponds to an *in vitro* translation product for snowdrop lectin. The 3' untranslated region contains six in-frame termination codons and one possible polyadenylation signal at position 532. Comparison of the aminoterminal sequence for the lectin and the deduced amino acid sequence for the lectin clone shows that the lectin is synthesized with a leader (signal) sequence of 23 amino acids (2315 daltons). It is also probable that 22 amino acids (2278 daltons) are removed post translationally from the C-terminal end of the protein.

The pea lectin A pre-pro-protein gene (LecA) encoding sequence in a form useful for the expression of LecA in transgenic plants may be derived from the pea genomic DNA-containing recombinant bacteriophage  $\lambda$ LecA [Gatehouse *et al*, Nucleic Acids Research (1987) 18, 7642].

The required fragment was isolated by polymerase chain reaction amplification of  $\lambda$ LecA DNA using the synthetic oligonucleotide:

5' -GACTCTAGAATGGCTTCTCTTCAAACC

as the N-terminal primer; and

5' -GACGGTACCCTATGCATCTGCAGCTTG

as the C-terminal primer. These 27-mers contain additional sequences at their 5' ends to introduce a restriction endonuclease *Xba*1 recognition sequence at the 5'-end and a *Kpn*1 recognition sequence at the 3'-end of the amplified pea sequence. This fragment was blunt-end ligated into the *Hind*III site of plasmid pUC18 to yield plasmid pVINp1. The lectin precursor encoding sequence is prepared from *Kpn*1 + *Xba*1 digested pVINp1 DNA and ligated into the *Kpn*1 + *Xba*1 digested binary vector pRok2 to yield the plasmid pRokVINp1. Plasmid pRokVINp1 is used to produce transgenic plants which express pea lectin A by conventional *Agrobacterium* mediated plant transformation.

The following Examples illustrate but do not limit the invention.

### **Example 1**

#### **Effect of lectins on in vitro mobility of cyst nematodes**

Cysts of the potato cyst nematode (PCN) *Globodera rostochiensis* were stimulated to hatch using potato root diffusate and newly emerged J2 juveniles were hand-picked for study. Groups of 10 J2 were transferred into watch glasses containing 1 ml of water, as a control, or aqueous solutions of phosphate buffer pH 6.4 or lectin in phosphate buffer. Lectins extracted from *Galanthus nivalis* (GNA), *Narcissus pseudonarcissus* (NPA) and *Pisum sativum* (Plec) were tested. Each treatment was replicated five times. J2 mobility was monitored every 12 hours for

three days in a controlled environment room at 12°C. J2 were considered immobile if they failed to respond to stimulation with a bristle [Alphey, Robertson & Lyon (1988) *Revue de Nématologie* 11 (4), 399-404].

The results are summarised in Table 1. It is clear that at all concentrations tested, all three lectins have a negative effect on PCN mobility.

Table 1. <i>In vitro</i> mobility test			
Concentration $\mu\text{g ml}^{-1}$	% Nematodes immobile after 72 hours		
	GNA	Plec	NPA
1	-	-	32
2	83	78	-
10	-	-	22
20	78	83	-
100	-	-	47
200	83	89	-

## **Example 2**

### **Effect of drench application of lectins on gall development by root-knot nematodes on tomato plants**

Glass tubes (7.5 x 2.5 cm) were each filled with 24.5g of sieved, dried sand, 1 ml of water containing c. 350 *Meloidogyne incognita* J2 juveniles and 5 ml of water or phosphate buffer pH 6.4 or solutions in phosphate buffer pH 6.4 of lectins to produce final concentrations of 0.1 to 100  $\mu\text{g ml}^{-1}$ . Each treatment was replicated 10 times. A 2 week old tomato seedling (cv. Moneymaker) was planted in each tube and after 14 days in a glasshouse at 22-27°C the roots were washed and the number of galls induced by nematode feeding recorded.

The results are summarised in Table 2. All three lectins give a reduction in the number of nematode galls formed on tomato seedlings.

Table 2. Drench test			
Concentration $\mu\text{g ml}^{-1}$	% Reduction of <i>M. incognita</i> galls on tomatoes		
	GNA	Plec	NPA
0.1	25	-	-
1	48	31	92
10	43	33	86
100	50	48	84

### Example 3

#### Construction and transformation of Snowdrop lectin clones

The LECGNA2 clone contained a 570 base *EcoR*I linked snowdrop lectin (GNA) gene cDNA cloned into the phagemid pT7T3 18U. The N-terminal and C-terminal peptides that are cleaved during processing to form the mature protein were marked on the sequence data.

The coding region of the lectin gene was subcloned into pUC19 using standard polymerase chain reaction (PCR) technology [Innis, M.A. *et al* eds. PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego. 1990]. Oligonucleotide primers were made covering the N-terminal and C-terminal regions which incorporated restriction sites so that the resultant amplified fragments could be subcloned using a *Bam*HI/*Kpn*I double digest. These primers comprised the sequences:

N-terminus: 5' -CGGATCCATGGCTAAGGCAAGT

C-terminus: 5' -CGGTACCTCATTACTTTGCCGT

Fragments were amplified using PCR and the LECGNA2 DNA as a template. The amplified fragments were cloned into pUC19 which had been linearised with *Bam*HI + *Kpn*I. Recombinant



plasmids were screened for the correct insert size with *Bam*HI/*Kpn*l. The resultant constructs (p1GNA2) were sequenced to ensure that no unwanted mutations had been created as artifacts of the PCR reaction.

The GNA encoding fragment was isolated by digestion of the p1GNA2 construct with *Bam*HI/*Kpn*l, ligated into *Bam*HI/*Kpn*l digested pROK2 and used to transform *E. coli* strain MC1022. These recombinants provided the *Agrobacterium* binary vector constructs which are useful for the constitutive expression of GNA in transgenic plants, illustrated in Figure 1. Colonies were screened by restriction digestion using *Bam*HI/*Kpn*l, *Sph*I and *Hind*III, and the correct p15GNA1 construct was mobilised into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating with HB101(pRK2013) according to established methods [Bevan, M. (1984) *Nucleic Acids Research*, 12, 103-110]. Single colonies containing the p15-GNA1 plasmids were rescreened by digestion with *Bam*HI/*Kpn*l to check for the correct insert size.

#### **Example 4**

##### **Construction of plasmids pGNA2 and pGNA3**

Plasmid p1GNA2 was digested with *Bam*HI and *Kpn*l and ligated to *Kpn*l-digested pAPT9 in the presence of an 8 base pair oligonucleotide (5'-GATCGTAC-3') used to link the *Kpn*l site and the *Bam*HI site at the 5' end of the inserted fragment. Following the transformation of *E. coli* MC1002 to ampicillin resistance, restriction analysis and sequencing were used to confirm that the fragment was inserted in the sense orientation and the correct presence of the linker oligo. The resultant plasmid, which was named pGNA1, carries a plant expression cassette comprising the CaMV35S promoter, the GNA coding region and the NOS terminator. This plasmid was digested

with *Bam*HI, ligated with *Bam*HI-digested pAPT5 and used to transform MC1022 to tetracycline resistance. Restriction and PCR-based analyses of the resultant constructs indicated that the CaMV35S - GNA - NOS cassette was inserted into pAPT5 in both orientations relative to the T-DNA, such that the GNA gene was transcribed towards the left border in plasmid pGNA2 and towards the right border in plasmid pGNA3. Plasmid pAPT5 is a pRK290-based binary vector that encodes tetracycline-resistance and carries a T-DNA region comprising two genes for plant selection: TR 2' promoter -  $\beta$ -glucuronidase (*uidA*) coding sequence - NOS terminator and CaMV 35S promoter - neomycin phosphotransferase (*aph3''II*) coding sequence - octopine synthase terminator. These genes are positioned such that the promoter sequences are adjacent and the *uidA* gene is proximal to the T-DNA left border sequence. Unique sites for *Hind*III, *Pac*I and *Bam*HI are also located within the T-DNA region and proximal to the right border sequence. Plasmids pGNA2 and pGNA3 were mobilised into *Agrobacterium tumefaciens* LBA4404 by triparental matings with *E.coli* HB101(pRK2013) according to established methods, selected on minimal agar containing tetracycline (1 mg/l) and the resultant single colonies streaked to purity on the same media. The presence of the correct plasmid, pGNA2 or pGNA3, in the resultant *Agrobacterium tumefaciens* LBA4404 strains was confirmed by restriction and PCR-based analyses.

### **Example 5**

#### **Construction of plasmid pPCG6**

Plasmid pPCG6 carries two insect-resistance genes: the GNA gene encoding a mannose-specific lectin from snowdrop (*Galanthus nivalis*, L) and the cowpea trypsin inhibitor (CpTI) gene isolated from cowpea (*Vigna unguiculata* Walp). The CpTI gene used was truncated at the 5' end such that it contains nucleotides +153 to +476 of the original CpTI sequence [Hilder, V. *et*

al, (1989) Plant Molecular Biology 13, 701-710] and the coding region starting at the second in-frame initiation codon. Standard PCR techniques were used to add *Bam*HI and *Kpn*I restriction sites at the 5' and 3' end of the coding region, respectively, allowing the fragment to be cloned into pUC19 between the same sites. This truncated CpTI coding region was subsequently excised using *Bam*HI and *Sst*I and cloned into *Bgl*II and *Sst*I sites located between the CaMV 35S promoter (-420 base pairs) and NOS terminator. The resultant CaMV 35S - CpTI - NOS cassette is contained on a 1.1 kb *Bam*HI fragment. As described in Example 4, the coding region of the GNA gene was subcloned from LECGNA2 into pGNA1, the resultant CaMV 35S - GNA - NOS cassette being contained on a 1.2 kb *Bam*HI fragment. To create pPCG6, a specially-designed polylinker was used containing a 150 bp "spacer" region derived from an upstream region (-433 to -583) of the CaMV 35S promoter bounded by *Bam*HI-*Bgl*II and *Bcl*I-*Bam*HI sites. The *Bam*HI fragments carrying the CpTI and GNA genes were cloned between the *Bam*HI-*Bgl*II sites and *Bcl*I-*Bam*HI sites, respectively, such that the two genes were oriented as a "head-to-head" inverted repeat. This conformation of genes limits the possibility of deletions occurring should any recombination take place between the similar sequences of the two cassettes, and should allow double-enhancement of the CaMV 35S promoters due to the close proximity of the two sequences. Finally, the 2.3 kb *Bam*HI fragment carrying both expression cassettes was cloned into the *Bam*HI site of pAPT5 such that the GNA gene was proximal to the right border sequence. Other details of pAPT5 are as described in Example 4.

#### Example 6

#### Transformation of tobacco

Transformation of tobacco *Nicotiana tabacum* var Samsun with *Agrobacterium tumefaciens* LBA4404 carrying p15GNA1 plasmids was carried out using the standard leaf disc method

[Horsch, R.B. *et al* (1985) *Science* 227, 1229-1231]. Leaf discs were cultured on selective media containing kanamycin at 100mg/l to select for transformed shoots. Shoots were rooted on kanamycin to eliminate untransformed escapes. Transformed plantlets were tested for snowdrop lectin expression by standard ELISA methods [Engvall, E. (1990) *Meths. Enzymol.* 70, 419]. Transgenic plants from lines 15GNA33, 15GNA35 and 15GNA79, express high levels of GNA antigen, equivalent to 40.2, 26.6 and 47.3 µg/g fresh weight respectively. The biological activity of the lectin in these plants may be demonstrated by standard haemagglutination assay procedures using trypsinised rabbit erythrocytes [Liss, H. & Sharon, N. (1973) *Ann. Rev. Biochem.* 42, 541-574] on phosphate buffered saline extracts of free-dried leaf tissue.

#### **Example 7**

##### **Transformation of potato**

Transformation of potato *Solanum tuberosum* cv. Désirée with *Agrobacterium tumefaciens* LBA4404 carrying pGNA2 plasmids was carried out using a stem section transformation method [Newell, C.A. *et al.* (1991) *Plant Cell Rep.* 10, 30-34]. Stem sections were cultured on selective media containing 100 mg/l kanamycin to select for transformed shoots. Shoots were rooted on kanamycin, and assayed for activity of the β-glucuronidase enzyme to identify transgenic shoots from non-transformed escapes. Transformed plantlets were tested by enhanced chemiluminescence for snowdrop lectin expression.

**Example 8****Transformation of tomato**

Transformation of tomato *Lycopersicon esculentum* cv. Ailsa Craig with *Agrobacterium tumefaciens* LBA4404 carrying pGNA2 plasmids was carried out, using stem sections from *in vitro* grown plantlets [Bird, C.R. *et al.* (1988) Plant Mol. Biol. **11**, 651-662]. Explants were cultured on media containing 50 mg/l kanamycin to select for transformed shoots. Shoots were rooted on kanamycin, which effectively eliminated non-transgenic escapes. Transformed plantlets were assayed for snowdrop lectin expression by standard, immuno-detection techniques using enhanced chemiluminescence. Transgenic plants expressed GNA protein up to a level of 0.4 % of total protein.

**Example 9****Transformation of oilseed rape**

Transformation of several lines of oilseed rape *Brassica napus* with *Agrobacterium tumefaciens* LBA4404 carrying pGNA2, pGNA3 or pPCG6 plasmids was carried out using a seedling hypocotyl method [de Block, M. *et al.* (1989) Plant Physiol. **91**, 694-701] Hypocotyl explants were cultured in the presence of 20 mg/l kanamycin to select for transformed shoots; shoots were rooted in medium containing kanamycin at the same level, which effectively screened out non-transgenic escapes. Plantlets were assayed for snowdrop lectin expression by standard, immuno-detection techniques using enhanced chemiluminescence. Transgenic lines expressed levels of GNA protein up to 1% of the total protein.

### **Example 10**

#### **Transformation of lettuce**

Transformation of lettuce with *Agrobacterium tumefaciens* LBA4404 carrying p15GNA1 plasmids was carried out using seedling cotyledons as the starting material [Micheltore, C. *et al.* (1987) Plant Cell Rep. 6, 439-442]. Cotyledon pieces were cultured in the presence of 50 mg/l kanamycin to select for transformed tissue. Shoots were rooted in medium containing the same level of kanamycin to screen out non-transgenic escapes. Transformed plantlets were assayed for snowdrop lectin expression by standard, immuno-detection techniques using enhanced chemiluminescence. Transgenic lines expressed levels of GNA protein up to approximately 2% of the total protein.

### **Example 11**

#### **Transformation of rice**

Transformation of rice (*Oryza sativa*) was achieved following micro-projectile bombardment using embryogenic suspension cultured cells as starting material [Cao, J *et al.* (1992) Plant Cell Reports 11:586-591]. Tungsten micro-projectiles were coated with 5 µg of plasmid DNA carrying the GNA coding region expressed from a suitable promoter; for instance, the Cauliflower mosaic virus 35S promoter or the maize *adh-1* promoter with the 5' intron sequence. Bombarded cells were selected using an appropriate agent; for example, cells bombarded with constructs expressing the coding region of the *Streptomyces hygroscopicus* phosphinothricin acetyl transferase gene (*bar*) are selected in the presence of 4mg/l glufosinate ammonium, or cells

expressing the hygromycin phosphotransferase gene (*hpt*) can be selected using hygromycin B at 25-50 mg/l. Transformed plants were regenerated from embryogenic calli and the expression of the snowdrop lectin gene assayed using standard, immunodetection techniques using enhanced chemiluminescence.

### **Example 12**

#### **Effect of transgenic oilseed rape expressing lectin and protease inhibitor genes on a cyst nematode, *Heterodera schachtii***

Lines of transgenic oilseed rape were generated which express the *Galanthus nivalis* lectin gene alone (GNA2) or in combination with the trypsin inhibitor gene from *Vigna unguiculata* (PCG6) as described in Example 9. Both transgenes were expressed from the Cauliflower mosaic virus 35S promoter. In all lines the T-DNA also carried the marker gene encoding  $\beta$ -glucuronidase. For each transgenic and control untransformed line, several replicate pots of two seeds each were planted in soil and grown under controlled environment glasshouse conditions. Where both seeds in the pot germinated, one was removed. When the plants were approximately 10 cm in height, a leaf disc was taken from each plant for assessment of  $\beta$ -glucuronidase activity; any plants failing to show activity were discarded. For each line, six transgenic and six control plants of approximately the same height were inoculated by pipetting a suspension containing 1000-1500 *Heterodera schachtii* eggs and infective juveniles into a 2-3 cm hole in the soil made adjacent to the developing root system. The plants were allowed to grow for 40 days before the root system of each plant was harvested for analysis. The female nematodes were washed from the root system and counted. Staining of the root system determined that the majority of the females in both control and transgenic lines were mature at the time of harvesting.

The mean number of females per root system is summarised in Table 3. It is clear that for both constructs used, the expression of the transgenes not only reduces the number of nematodes per root system, but also reduces the percentage of mature female nematodes in the population.

Table 3. Mean number of female <i>Heterodera schachtii</i> per oilseed rape root system						
LINE	PCG6, line 492		PCG6, line 453		GNA2, line 254	
	control	transgenic	control	transgenic	control	transgenic
Mean females per root	907.8	424.8	565.6	439.0	213.6	148.6
SD	171.0	106.7	151.6	88.7	49.1	96.9
% reduction over control		53.2		22.4		30.5

PCG6: transgenic plants expressing lectin and protease inhibitor genes.

GNA2: transgenic plants expressing lectin gene.

### **Example 13**

#### **Effect of transgenic oilseed rape expressing lectin and protease inhibitor genes on a migratory endoparasitic nematode, *Pratylenchus neglectus***

The lines of transgenic oilseed rape used were as described in Example 12. Seeds were surface sterilised, germinated under sterile conditions on agar-containing media and allowed to grow for 10 days. A sample of plant material was tested for  $\beta$ -glucuronidase activity and any plants showing no activity were discarded. For each line tested, between four and seven transgenic and control plants were inoculated with  $122 \pm 11$  active *Pratylenchus neglectus* nematodes



applied directly to the agar surface. After 3 months, the number of nematodes per plant was counted.

The data summarised in Table 4 clearly indicates that for both transgenic lines PCG6 and GNA2, the number of nematodes per root is severely reduced.

Table 4. Mean numbers of <i>Pratylenchus neglectus</i> nematodes per oilseed rape root system.				
Line	PCG6, line 492		GNA2, line 419	
	control	transgenic	control	transgenic
Mean number of nematodes per root	1821.4	542.6	178.7	98.7
SD	729.8	492.6	72.8	176.9
% reduction over control		70		45

**Example 14****Effect of transgenic potato expressing a lectin gene on a cyst nematode, *Globodera pallida***

Lines of transgenic potato were generated which express the *Galanthus nivalis* lectin gene (GNA2) as described in Example 7. The transgene was expressed from the Cauliflower mosaic virus 35S promoter. For each transgenic and control untransformed line, several replicate 60ml canisters containing soil were planted with a potato tuber and inoculated with 1500 *Globodera pallida* eggs and infective juveniles. The canisters were capped and incubated in the dark for four weeks at 18°C. The root system of each potato plant was harvested and the number of nematode cysts counted. The mean number of cysts per plant root system is summarised in Table 5. It is clear that the transgenic lines expressing the lectin gene have significantly fewer nematode cysts than the untransformed lines.

Table 5. Mean number of <i>Globodera pallida</i> cysts per potato root system				
LINE	GNA2, Line 23	GNA2, Line 67	GNA2 Line 73	Untransformed Control
Mean cysts per root system	0.25	0.25	0.33	20 - 30
% reduction over control	98.8 - 99.2	98.8 - 99.2	98.3 - 98.9	-

SEQUENCE ID NO: LECGNA1  
 SEQUENCE TYPE: Nucleotide sequence with corresponding protein  
 SEQUENCE LENGTH: 610 bases  
 STRANDEDNESS: Double-stranded  
 TOPOLOGY: Linear  
 MOLECULE TYPE: cDNA t mRNA  
 ORIGINAL SOURCE ORGANISM: *Galanthus nivalis*  
 EXPERIMENTAL SOURCE: Clones  
 FEATURES: from 1 to 67 bp putative signal peptide P  
 from 68 to 382 bp putative mature protein P  
 from 383 to 487 bp putative C-terminal peptide P  
 from 488 to 610 bp 3' untranslated region P

G GCT AAG ACA ATT CTC CTC ATT TTG GCC ACC ATC TTC CTT GGT GTC ATC 49  
 Ala Lys Thr Ile Leu Leu Ile Leu Ala Thr Ile Phe Leu Gly Val Ile  
 -20 -15 -10  
 ACA CCA TCT TGC CTG AGT AAT AAT ATC CTG TAC TCT GGC GAG ACT CTC 97  
 Thr Pro Ser Cys Leu Ser Asn Asn Ile Leu Tyr Ser Gly Glu Thr Leu  
 -5 1 5 10  
 TCT GCC GGC GAA TTT CTC AAC CAA GGC AAT TAT GTT TTT ATC ATG CAA 145  
 Ser Ala Gly Glu Phe Leu Asn Gln Gly Asn Tyr Val Phe Ile Met Gln  
 15 20 25  
 GAG GAC TGC AAT CTG GTC TTG TAC GAC GTT GAC AAG CCT CTC TGG GAA 193  
 Glu Asp Cys Asn Leu Val Leu Tyr Asp Val Asp Lys Pro Leu Trp Glu  
 30 35 40  
 ACA AAC ACA GGC GGC CTC TCC CGT CGC TGC TAT CTC AAC ATG CAG ACT 241  
 Thr Asn Thr Gly Gly Leu Ser Arg Arg Cys Tyr Leu Asn Met Gln Thr  
 45 50 55  
 GAT GGG AAC CTC GTC GTG TAC AAC CCG TCG AAC AAA CCG ATT TGG GCA 289  
 Asp Gly Asn Leu Val Val Tyr Asn Pro Ser Asn Lys Pro Ile Trp Ala  
 60 65 70  
 AGC AAC ACT GGA GGC CAG AAT GGT AAT TAT GTG TGC ATC CTT CAG AAG 337  
 Ser Asn Thr Gly Gly Gln Asn Gly Asn Tyr Val Cys Ile Leu Gln Lys  
 75 80 85 90  
 GAT GGG AAC ATT GCG ATC TAC GGA CCT GCT ATT TGG GCT ACT GGA ACC 385  
 Asp Gly Asn Ile Ala Ile Tyr Gly Pro Ala Ile Trp Ala Thr Gly Thr  
 95 100 105  
 AAT ATT CAT GGA GCT GGA ATA GTT GGA GTT CTT GGA TCA GCA CCA CAG 433  
 Asn Ile His Gly Ala Gly Ile Val Gly Val Leu Gly Ser Ala Pro Gln  
 110 115 120  
 AAT TCT ACT GCT GAA ATG ATA AAG CTA GTG AGG AAG TAC CTA ATC ACT 481  
 Asn Ser Thr Ala Glu Met Ile Lys Leu Val Arg Lys Tyr Leu Ile Thr  
 125 130 140  
 AAG TAA TTATGACCCG TGAGGTCCGG ACTGCATGTT TGTGAGAATG AGGAATAAAA 537  
 Lys  
 GTCCAACCAT GTGGTGGACT CCTGAAAATA AATAACTGCT ATGTATGATG TAATGGAGAC 597  
 TTATCTACTT TGC 610

SEQUENCE ID NO: LECGNA2  
 SEQUENCE TYPE: Nucleotide sequence with corresponding protein  
 SEQUENCE LENGTH: 570 bases  
 STRANDEDNESS: Double-stranded  
 TOPOLOGY: Linear  
 MOLECULE TYPE: cDNA to mRNA  
 ORIGINAL SOURCE ORGANISM: *Galanthus nivalis*  
 EXPERIMENTAL SOURCE: Clones  
 FEATURES: from 1 to 17 bp 5' untranslated region E  
 from 18 to 86 bp signal peptide E  
 from 87 to 401 bp mature protein E  
 from 402 to 491 bp C-terminal peptide E  
 from 492 to 570 bp 3' untranslated region E

CAACTACAAG TTACAAA ATG GCT AAG GCA AGT CTC CTC ATT TTG GCC GCC ATC	53
Met Ala Lys Ala Ser Leu Leu Ile Leu Ala Ala Ile	
-20 -15	
TTC CTT GGT GTC ATC ACA CCA TCT TGC CTG AGT GAC AAT ATT TTG TAC	101
Phe Leu Gly Val Ile Thr Pro Ser Cys Leu Ser Asp Asn Ile Leu Tyr	
-10 -5 1 5	
TCC GGT GAG ACT CTC TCT ACA GGG GAA TTT CTC AAC TAC GGA AGT TTC	149
Ser Gly Glu Thr Leu Ser Thr Gly Glu Phe Leu Asn Tyr Gly Ser Phe	
10 15 20	
GTT TTT ATC ATG CAA GAG GAC TGC AAT CTG GTC TTG TAC GAC GTG GAC	197
Val Phe Ile Met Gln Glu Asp Cys Asn Leu Val Leu Tyr Asp Val Asp	
25 30 35	
AAG CCA ATC TGG GCA ACA AAC ACA GGT GGT CTC TCC CGT AGC TGC TTC	245
Lys Pro Ile Trp Ala Thr Asn Thr Gly Gly Leu Ser Arg Ser Cys Phe	
40 45 50	
CTC AGC ATG CAG ACT GAT GGG AAC CTC GTG GTG TAC AAC CCA TCG AAC	293
Leu Ser Met Gln Thr Asp Gly Asn Leu Val Val Tyr Asn Pro Ser Asn	
55 60 65	
AAA CCG ATT TGG GCA AGC AAC ACT GGA GGC CAA AAT GGG AAT TAC GTG	341
Lys Pro Ile Trp Ala Ser Asn Thr Gly Gly Gln Asn Gly Asn Tyr Val	
70 75 80 85	
TGC ATC CTA CAG AAG GAT AGG AAT GTT GTG ATC TAC GGA ACT GAT CGT	389
Cys Ile Leu Gln Lys Asp Arg Asn Val Val Ile Tyr Gly Thr Asp Arg	
90 95 100	
TGG GCT ACT GGA ACT CAC ACC GGA CTT GTT GGA ATT CCC GCA TCG CCA	437
Trp Ala Thr Gly Thr His Thr Gly Leu Val Gly Ile Pro Ala Ser Pro	
105 110 115	
CCC TCA GAG AAA TAT CCT ACT GCT GGA AAG ATA AAG CTT GTG ACG GCA	485
Pro Ser Glu Lys Tyr Pro Thr Ala Gly Lys Ile Lys Leu Val Thr Ala	
120 125 130	
AAG TAA TGACCGGTGA TCTTTTAACT TGCATGTATG TGGAAGAGT AATAAAATAA	541
Lys	
GTGCATTGTA GATAATCGAC CTCGTCGCG	570

SEQUENCE ID NO: LECGNA3  
 SEQUENCE TYPE: Nucleotide sequence with corresponding protein  
 SEQUENCE LENGTH: 667 bases  
 STRANDEDNESS: Doubl -stranded  
 TOPOLOGY: Linear  
 MOLECULE TYPE: cDNA to mRNA  
 ORIGINAL SOURCE ORGANISM: *Galanthus nivalis*  
 EXPERIMENTAL SOURCE: Clones  
 FEATURES: from 1 to 62 bp putative signal peptide P  
 from 63 to 377 bp putative mature protein P  
 from 378 to 467 bp putative C-terminal peptide P  
 from 468 to 667 bp 3' untranslated region P

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Thr Ile Leu Leu Ile Leu Ala Thr Ile Phe Leu Gly Val Ile Thr Pro	
-15 -10 -5	
TCT TGC CTG AGT GAA AAT GTT CTG TAC TCC GGT GAG ACT CTC CCT ACA	98
Ser Cys Leu Ser Glu Asn Val Leu Tyr Ser Gly Glu Thr Leu Pro Thr	
1 5 10	
GGG GGA TTT CTC TCC TCT GGC AGT TTT GTT TTT ATC ATG CAA GAG GAC	146
Gly Gly Phe Leu Ser Ser Gly Ser Phe Val Phe Ile Met Gln Glu Asp	
15 20 25	
TGC AAC CTG GTC CTG TAC AAC GTC GAC AAG CCC ATC TGG GCA ACT AAC	194
Cys Asn Leu Val Leu Tyr Asn Val Asp Lys Pro Ile Trp Ala Thr Asn	
30 35 40	
ACA GGC GGC CTC TCC AGT GAC TGC ACC CTC AGC ATG CAG ACC GAT GGG	242
Thr Gly Gly Leu Ser Ser Asp Cys Thr Leu Ser Met Gln Thr Asp Gly	
45 50 55 60	
AAC CTC GTA GTG TAC ACC CCA TCG AAC AAA CCG ATT TGG GCA AGC AAC	290
Asn Leu Val Val Tyr Thr Pro Ser Asn Lys Pro Ile Trp Ala Ser Asn	
65 70 75	
ACT GAC AGC CAG AAT GGG CAT TAC GTG TGC ATC CTT CAA AAG GAT CGG	338
Thr Asp Ser Gln Asn Gly His Tyr Val Cys Ile Leu Gln Lys Asp Arg	
80 85 90	
AAC GTT GTG ATC TAC GGA ACT GAT CGT TGG GCT ACA GGA ACT TAC ACC	386
Asn Val Val Ile Tyr Gly Thr Asp Arg Trp Ala Thr Gly Thr Tyr Thr	
95 100 105	
GGT GCT GTT GGA ATT CCT GAA TCA CCC CCC TCG GAG AAA TAT CCT ACT	434
Gly Ala Val Gly Ile Pro Glu Ser Pro Pro Ser Glu Lys Tyr Pro Thr	
110 115 120	
GCT GGA AAG ATA AAG CAA GTG ACC GAA AAG TAA TGACCGGTGA TCTATGAACC	487
Ala Gly Lys Ile Lys Gln Val Thr Glu Lys	
130 135	
TTGCATGCAT GTGAGAAGAG TAATATAATA TATGTGCATT TTAGATCAAT GCACACGGTG	547
TTTCTTTGTC ACAAATAAAT AACTAGGTTG TACTGGACGT AAATAAAGTC CGGCCTCCTA	607
GTTTCGTGCC TTGTACGCAT CTTGTACGCA TCTTGTATGC ATGCATTTTG GAAAGGAGGC	667

SEQUENCE ID NO: LECGNA5  
 SEQUENCE TYPE: Nucleotide sequence with corresponding protein  
 SEQUENCE LENGTH: 650 bases  
 STRANDEDNESS: Double-stranded  
 TOPOLOGY: Linear  
 MOLECULE TYPE: cDNA t mRNA  
 ORIGINAL SOURCE ORGANISM: *Galanthus nivalis*  
 EXPERIMENTAL SOURCE: Clones  
 FEATURES: from 1 to 63 bp putative signal peptide P  
 from 64 to 378 bp putative mature protein P  
 from 379 to 468 bp putative C-terminal peptide P  
 from 469 to 650 bp 3' untranslated region P

AAG ACA AGT CTC CTC ATT TTG GCC ACC ATC TTC CTT GGA GTC ATC GCA	48
Lys Thr Ser Leu Leu Ile Leu Ala Thr Ile Phe Leu Gly Val Ile Ala	
-20 -15 -10	
CCA TCT TGC CTG AGT GAA AAT ATT CTG TAC TCC GGT GAG ACT CTC CCT	96
Pro Ser Cys Leu Ser Glu Asn Ile Leu Tyr Ser Gly Glu Thr Leu Pro	
-5 1 5 10	
ACA GGG GGA TTT CTC TCC TCT GGC AGT TTT GTT TTT ATC ATG CAA GAG	144
Thr Gly Gly Phe Leu Ser Ser Gly Ser Phe Val Phe Ile Met Gln Glu	
15 20 25	
GAC TGC AAC CTG GTC TTG TAC AAC GTC GAC AAG CCC ATC TGG GCA ACT	192
Asp Cys Asn Leu Val Leu Tyr Asn Val Asp Lys Pro Ile Trp Ala Thr	
30 35 40	
AAC ACT GGT GGC CTC TCC AGT GAC TGC TCC CTC AGC ATG CAG ACA GAT	240
Asn Thr Gly Gly Leu Ser Ser Asp Cys Ser Leu Ser Met Gln Thr Asp	
45 50 55	
GGG AAC CTC GTA GTG TAC ACC CCA TCG AAC AAA CCG ATT TGG GCA AGC	288
Gly Asn Leu Val Val Tyr Thr Pro Ser Asn Lys Pro Ile Trp Ala Ser	
60 65 70 75	
AAC ACT GAC GGC CAG AAT GGG AAT TAC GTG TGC ATC CTT CAA AAG GAT	336
Asn Thr Asp Gly Gln Asn Gly Asn Tyr Val Cys Ile Leu Gln Lys Asp	
80 85 90	
CGG AAC GTT GTG ATC TAC GGA ACT AAT CGT TGG GCT ACT GGA ACT CAC	384
Arg Asn Val Val Ile Tyr Gly Thr Asn Arg Trp Ala Thr Gly Thr His	
95 100 105	
ACC GGT GCT GTA GGA ATT CCT GCA TCA CCG CCC TCG GAG AAA TAT CCT	432
Thr Gly Ala Val Gly Ile Pro Ala Ser Pro Pro Ser Glu Lys Tyr Pro	
110 115 120	
ACT GCT GGA ATG ATA AAG CAA GTG ACC GAA AAG TAA TGACCGGTGG	478
Thr Ala Gly Met Ile Lys Gln Val Thr Glu Lys	
TGATCTATGA ACCTTG CATG CATGTGAGAA GAGTAATAAA ATATGTGCAT TTTAGATCAA	538
TGCACACGGT GTTTGTTTGT CACAAATAAA TAACTAGGTT GTACTGGACA TAAATATAGT	598
CCCGCCTCCT GGTTTCATGC CTTGTACGCA TCTTCTATGC ATGCATTTTG GA	650

SEQUENCE ID NO: LECGNAB  
 SEQUENCE TYPE: Nucleotide sequence with corresponding protein  
 SEQUENCE LENGTH: 597 bases  
 STRANDEDNESS: Double-stranded  
 TOPOLOGY: Linear  
 MOLECULE TYPE: cDNA to mRNA  
 ORIGINAL SOURCE ORGANISM: *Galanthus nivalis*  
 EXPERIMENTAL SOURCE: Clones  
 FEATURES: from 1 to 61 bp putative signal peptide P  
 from 62 to 376 bp putative mature protein P  
 from 377 to 481 bp putative C-terminal peptide P  
 from 482 to 597 bp 3' untranslated region P

G ACA AGT CTC CTC ATT TTG GCC ACC ATC TTC CTT GGA GTC ATC ACA CCA Thr Ser Leu Leu Ile Leu Ala Thr Ile Phe Leu Gly Val Ile Thr Pro -20 -15 -10 -5	49
TCT TGC CTG AGT GAT AAT ATT ATG TAC TCT GGC GAG ACT CTC TCT ACT Ser Cys Leu Ser Asp Asn Ile Met Tyr Ser Gly Glu Thr Leu Ser Thr 1 5 10	97
GGC GAA TTT CTC AAC TAC GGC AGT TAT GTT TTT ATC ATG CAA GAG GAC Gly Glu Phe Leu Asn Tyr Gly Ser Tyr Val Phe Ile Met Gln Glu Asp 15 20 25	145
TGC AAT CTG GTC TTG TAC GAC GTT GAC AAG CCT ATC TGG GCA ACA AAC Cys Asn Leu Val Leu Tyr Asp Val Asp Lys Pro Ile Trp Ala Thr Asn 30 35 40	193
ACA GGT GGC CTC TCC CGT AGC TGC TAT CTC AAC ATG CAG ACC GAC GGG Thr Gly Gly Leu Ser Arg Ser Cys Tyr Leu Asn Met Gln Thr Asp Gly 45 50 55 60	241
AAC CTC GTC GTG TAC AAC CCG TCG AAC AAA CCG ATT TGG GCA AGC AAC Asn Leu Val Val Tyr Asn Pro Ser Asn Lys Pro Ile Trp Ala Ser Asn 65 70 75	289
ACT GGA GGC CAG AAT GGT AAT TAT GTG TGC ATC CTT CAG AAG GAT CGG Thr Gly Gly Gln Asn Gly Asn Tyr Val Cys Ile Leu Gln Lys Asp Arg 80 85 90	337
AAC GTT GTG ATC TAC GGA CCT GCT CGT TGG GCT ACT GGA ACC AAT ATT Asn Val Val Ile Tyr Gly Pro Ala Arg Trp Ala Thr Gly Thr Asn Ile 95 100 105	385
CAT GGT GCT GGA ATA GTT GGA GTT CCT GGA TCA GCA CCA CAG AAT TCT His Gly Ala Gly Ile Val Gly Val Pro Gly Ser Ala Pro Gln Asn Ser 110 115 120	433
ACT GCT GAA ATG ATA AAG CTA GTG AGG AAG TAC CTA ATC ACT AAG TAA Thr Ala Glu Met Ile Lys Leu Val Arg Lys Tyr Leu Ile Thr Lys 125 130 135	481
TTATGACCCG TGAGGTCCCG GCTGCATGTG TGTGAGAATG AGGAATAAAA GTAAAACCAT GTGGTGGACG TGCTGAAAAT AAATAACTGC TATGTATGAT GTAATGGAGA CTTATC	541 597

**CLAIMS**

1. The use of lectins derived from Amaryllidaceae, Alliaceae, or Viciaeae for the control of nematodes.
2. Use according to claim 1, wherein the lectins are derived from *Allium sativum* (garlic), *Allium vineale*, *Allium ursinum*, *Allium moly*, *Allium cepa*, *Allium porrum*, *Narcissus pseudonarcissus*, *Clivia miniata*, *Galanthus nivalis* (snowdrop), *Hippeastrum hybr*, *Cicer* spp., *Lens culinaris*, *Lathyrus odoratus* and *Pisum sativum* (pea).
3. Use according to claim 1 or 2, in which the lectin derives from expression of the lectin gene in a transgenic plant.
4. Use according to claim 3, in which the lectin gene has a sequence identical or having substantial functional homology with one of the sequence listings LECGNA1, LECGNA2, LECGNA3, LECGNA5, and LECGNA8.
5. Use according to any of claims 1 to 4, for the control of nematodes of the class comprising the families Longidoridae, Trichodoridae, Anguinidae, Dolichodoridae, Belenolaimidae, Pratylenchidae, Hoplolaimidae, Heteroderidae, Criconematidae, Tylenchulidae, Aphelenchoididae and Fergusobiidae.
6. Use according to any of the preceding claims, for the protection of rice, wheat, maize, cotton, potato, sugarcane, grapevines, cassava, sweet potato, tobacco, soyabean, sugar beet, beans, banana, tomato, lettuce, oilseed rape and sunflower.



7. A method of controlling nematode pests in a host or an environment subject to nematode attack which comprises establishing in said host or environment a nematicidal amount of a lectin derived from Amaryllidacea, Alliacea, or Viciaeae.
8. A method according to claim 7, in which the host is genetically modified so as to present the lectin at the site of nematode attack.
9. A method according to claim 7 or 8, in which the host is a plant.

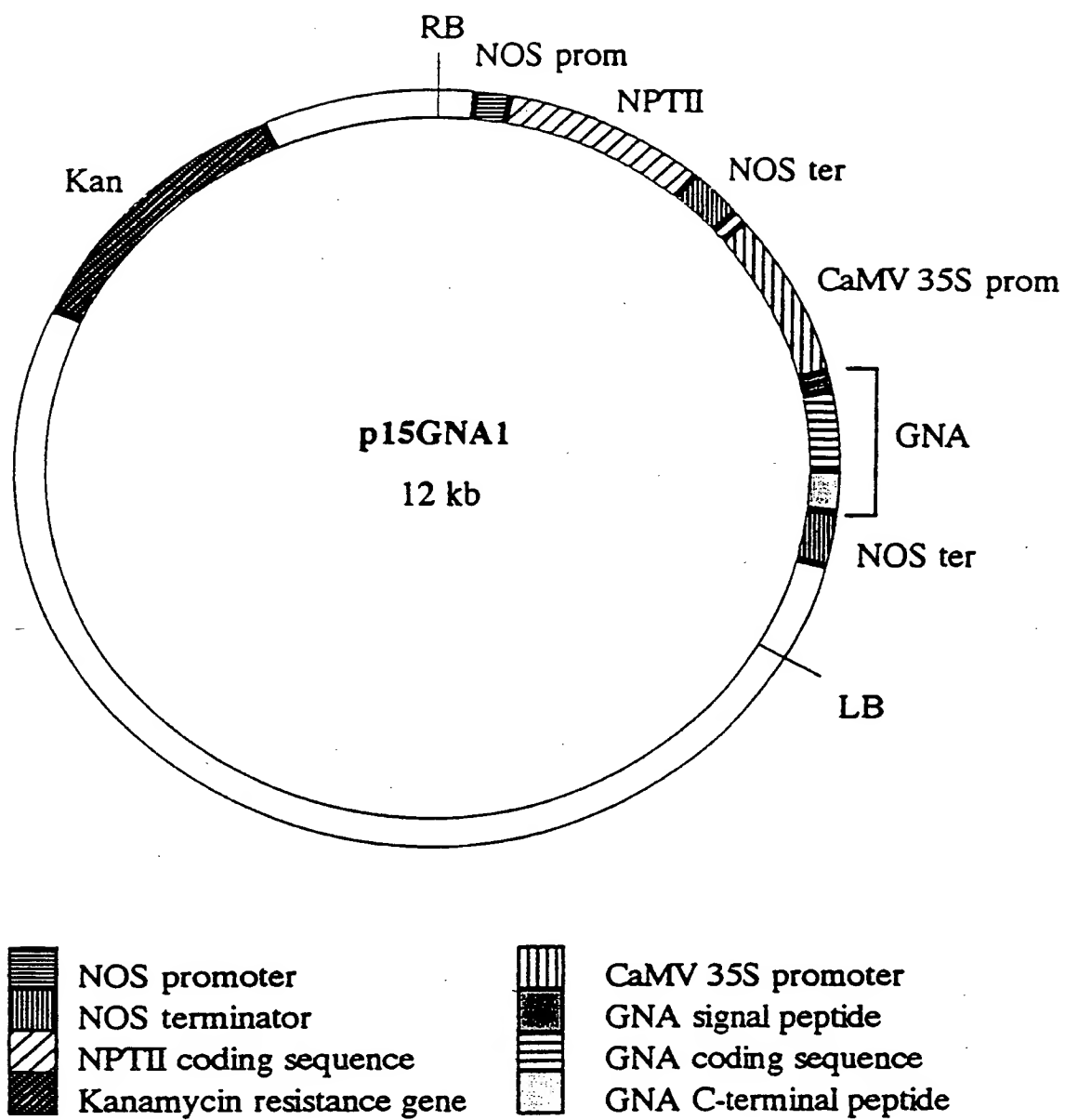


Figure 1

# INTERNATIONAL SEARCH REPORT

Inter nal Application No  
PCT/GB 95/00730

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A01N65/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 02139 ( AGRIC GENETICS CO LTD) 20 February 1992 cited in the application see the whole document ---	1-9
A	EP,A,0 427 529 ( PIONEER HI-BRED INT INC) 15 May 1991 cited in the application see the whole document ---	1-9
A	EP,A,0 351 924 ( SHELL INT RES MIJ BV) 24 January 1990 cited in the application see page 3, line 16-18 ---	1-9
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☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

24 July 1995

Date of mailing of the international search report

04.09.95

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 95/00730

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FUNDAM. APPL. NEMATOL., vol. 15, no. 2, 1992 pages 97-100, N. MARBAN-MENDOZA, M.B. DICKLOW & B.M. ZUCKERMAN 'Control of Meloidogyne inc. on tomato by two leguminous plants.' see page 99, column 1, paragraph 5 - column 2, paragraph 2 ---	1-9
A	J. NEMATOL., vol. 19, no. 3, 1987 pages 331-335, N. MARBAN-MENDOZA, A. JEYAPRAKASH, H.-B.JANSSON, R.A. DAMON & B.M. ZUCKERMAN. 'Control of root-knot nematodes on tomato by lectins.' see the whole document -----	1-9

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No  
PCT/GB 95/00730

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